# [ABSTRACT OF DISCLOSURE]

[Abstract]

The present invention relates to methods for precisely and effectively detecting mutations of organism.

[Representative Drawing]

FIG. 1

[SPECIFICATION]

[TITLE OF INVENTION]

METHOD FOR DETECTING BASE MUTATIONS

[BRIEF DESCRIPTION OF THE DRAWINGS]

Fig. 1 shows the MALDI-TOF mass spectrum of the 7mer when the 2741$^{st}$ base of the 4$^{th}$ intron of human maspin gene is normal (C/C).

Fig. 2 shows the MALDI-TOF mass spectrum of the 13mer when the 2741$^{st}$ base of the 4$^{th}$ intron of human maspin gene is normal (C/C).

Fig. 3 shows the MALDI-TOF mass spectrum of the 7mer when the 2741$^{st}$ base of the 4$^{th}$ intron of human maspin gene is hetero (C/T).

Fig. 4 shows the MALDI-TOF mass spectrum of the 13mer when the 2741$^{st}$ base of the 4$^{th}$ intron of human maspin gene is hetero (C/T).

Fig. 5 shows the MALDI-TOF mass spectrum of the 7mer when the 2741$^{st}$ base of the 4$^{th}$ intron of human maspin gene is all changed into T (T/T).

Fig. 6 shows the MALDI-TOF mass spectrum of the 13mer when the 2741$^{st}$ base of the 4$^{th}$ intron of human maspin gene is all changed into T (T/T).

Fig. 7 shows the MALDI-TOF mass spectrum of the 7mer and the 13mer when the 3597$^{th}$ base of the 4$^{th}$ intron of human maspin (serpinb5) gene is normal (C/C).

Fig. 8 shows the MALDI-TOF mass spectrum of the 7mer and the 13mer when the 3597$^{th}$ base of the 4$^{th}$ intron of human maspin (serpinb5) gene is hetero (C/T).

Fig. 9 shows the MALDI-TOF mass spectrum of the 7mer and the 13mer when the 3597$^{th}$ base of the 4$^{th}$ intron of human maspin (serpinb5) gene is all changed into T

(T/T).

[DETAILED DESCRIPTION OF THE INVENTION]

[OBJECT OF THE INVENTION]

[TECHNICAL FIELD OF THE INVENTION AND BACKGROUND ART OF THE FIELD]

Genetic analysis of an organism is used for disease risk, diagnosis, prognosis or disease treatment. For example, mutation analysis on a specific gene of a specific person makes it possible to predict the disease risk, thereby inducing prevention of the disease. The present invention relates to a method for analyzing genes of an organism, and more specifically to a method for determining genetic variation in an organism.

Human genome project enables more broad measurement of disease risk, diagnosis or prognosis and prediction of reaction on medication. Nucleotide sequence analysis of a plurality of individuals presents polymorphic sites, which are referred to as SNPs (single nucleotide polymorphisms). The SNP is a variation occurred over the specific frequency in a nucleotide sequence of chromosome in organism. In human body, SNPs occur every about 1,000 bases. In consideration of the size of human genome, millions of SNPs exist in human body. Since the SNP is regarded as a means for explaining characteristic difference between individuals, the SNP can be used in prevention or treatment of disease by examination of cause of disease.

SNPs discovered by the human genome project show only that the polymorphism exist in human body but do not show how those polymorphisms are related to disease. In order to reveal the relationship between the SNPs and diseases, a

comparative analysis of polymorphism pattern represented in healthy people and patients, SNP scoring, is required. For precise examination of the relationship between the SNP and disease, a large number of SNPs should be analyzed without error.

The SNP scoring method includes DNA sequencing, PCR-SSCP (Polymerase chain reaction – Single stranded conformation polymorphism), allele specific hybridization, oligo-ligation, mini-sequencing and enzyme cleavage method. A method using a DNA chip is also introduced, but it is not different from the allele specific hybridization in principle except that it uses a support to which oligonucleotide probe adheres.

The two classical methods for carrying out DNA sequencing are the Maxam and Gilbert chemical procedure and the Sanger method that has been mainly used in recent years. The DNA sequencing method is to find out nucleotide sequences of the whole or a part of genes rather than to examine genetic variations of specific sites. Since genetic variations of specific sites may be identified by examination of nucleotide sequences, the DNA sequencing method can be used for the SNP scoring. However, the DNA sequencing method is ineffective because adjacent nucleotide sequences that do not require examination are read with target SNP to be examined.

In PCR-SSCP (Orita, M. et al., Genomics, 1989, 5:8874-8879), sequences including SNPs to be analyzed are amplified by PCR, and then separated into each strand. Thereafter, electrophoresis is performed on polyacrylamide gel. Since the secondary structure of DNA strand is changed by difference of one base in sequence, the one base variation in sequence may be examined from differences in electrophoresis running velocity resulting from the difference of structure.

The allele specific hybridization is to examine variations by hybridizing DNAs labeled with radioisotope to probes attached to a nylon filer, by regulating hybridization conditions such as temperature.

The oligo-ligation (Nucleic Acid Research 24, 3728, 1996) is to examine sequence variations by performing a ligation reaction under a condition where the ligation does not happen if target DNA is non-complementary with template DNAs, followed by confirming whether it happens.

The mini-sequencing (Genome Research 7:606, 1997) is developed for SNP scoring. This method performs DNA polymerization in a condition that only one base of interest can be polymerized and distinguish what the polymerized base is.

The PCR-SSCP, the allele specific hybridization, the oligo-ligation are ineffective methods in analysis of many samples because of its use of polyacrylamide gel. And the errors resulting from mismatching of probes with undesired sites cannot be identified by those methods.

Although the mini-sequencing is simple and effective in analysis of many samples since it was developed for the SNP scoring, the incorrect result by errors of the mismatching cannot be still identified, and base deletion and insertion cannot be found by the mini-sequencing.

The enzyme cleavage method is also developed for SNP scoring (WO 01/90419). In the enzyme cleavage method, sequences to be analyzed are amplified by appropriate methods like the PCR. The amplified products include sequences that can be cleaved or recognized by two restriction enzymes. The enzyme cleavage method is to examine sequence variations by cleaving the amplified products with two restriction enzymes and

measuring the molecular weight of the cleaved fragments. The enzyme cleavage has an advantage of simplicity and rapidity because the molecular weight of the fragments obtained from the restriction enzyme reaction is measured by mass spectrometry right after amplification of genes by PCR. However, the incorrect analysis by errors is not identified by the enzyme cleavage method described in WO 01/90419. Although the incorrect analysis may be induced when primers are combined in undesired sites during the PCR, it is not identified. That is to say, the primer used to examine polymorphisms of CYP2C9 may be combined with CYP2C8. In this case, it is difficult to discover whether the errors are generated because whether the primer is combined with CYP2C8 other than CYP2C9 cannot be identified. Also, this method can detect one base substitution with other bases, but cannot detect base deletion or insertion.

[TECHNICAL SUBJECT TO BE ACHIEVED BY THE INVENTION]

In the present invention, there was developed a method for precisely and effectively detecting mutations of organism. That is to say, there was developed a method for precisely and effectively detecting mutations of organism by deletion or insertion, as well as searching an exact base mutation by enabling simple and rapid examination of mutations in many samples and precise examination of mutation by identification of errors resulting from binding of primers at incorrect regions, as well as detecting mutations by deletion or insertion.

[CONSTITUTION OF THE INVENTION]

There is provided a method for precisely and effectively detecting mutations of

organism in an embodiment of the present invention.

In order to analyze mutations, the method of present invention amplify desired sequence to include sites where the resulting product may be cleaved by restriction enzymes and the number of bases in the fragments cleaved by the restriction enzymes is designed to be 32 or less and at least one base among them is made to be produced by replication of template not primers itself and after the amplified fragments are cleaved by restriction enzymes, the molecular weight of the fragments is measured to analyze mutations.

In order to analyze mutations, the method of the present invention amplify desired sequence to include sites where the resulting product may be cleaved by restriction enzymes and the cleaved fragments have a following structure.

| 5'- | Primer binding sequence 1 | Restriction Enzyme recognition sequence | Primer binding sequence 2 | Front sequence from mutation | Mutation sequence | Sequence behind mutation | Primer binding sequence 3 | -3' |
|---|---|---|---|---|---|---|---|---|

The term 'restriction enzyme recognition sequence' is a sequence simultaneously or adjacently recognized by different restriction enzymes, which may not correspond to a cleaved sequence. For example, both Fok1 and BstF5I recognize the sequence GGATG. But, the cleaved sites are next to the $9^{th}/13^{th}$ and $2^{nd}/0^{th}$ bases from the 3' end of the recognition sequence, respectively. Both of the two restriction enzymes for recognizing the restriction enzyme recognition sequence may have the same optimum temperature or different optimum temperatures, both restriction enzymes being used in the present invention. Among them, the restriction enzymes having different optimum

temperatures are more preferable. Preferably, the restriction enzymes are a restriction enzyme having a relatively low optimum temperature selected from the group consisting of Fok1, Bbv I, Bsg I, Bcg I, Bpm I, BseR I and Bae I, and a restriction enzyme having a relatively high optimum temperature selected from the group consisting of BstF5 I, Taq I, BsaB I, Btr I, BstAP I, Fau I, Bcl I, Pci I and Apo I. Most preferably, the restriction enzymes are Fok1 and BstF5 I.

The restriction enzymes having the relatively low optimum temperature are Bae I(25°C), Fok1, Bbv I, Bsg I, Bcg I, Bpm I and BseR I (37°C). The enzymes having the relatively high optimum temperature are BstF5 I, Taq I (65°C), BsaB I, Btr I, BstAP I (60°C), Bcl I, Pci I and Apo I (50°C).

One of the two primers used in PCR amplification comprises a primer binding sequence 1, a restriction enzyme recognition sequence and a primer binding sequence 2, and the other primer comprises a primer binding sequence 3.

The 'primer binding sequence' is a sequence that is complementary with nucleic acid to be template, but the restriction enzyme recognition sequence may not be complementary with the nucleic acid. The number of bases of the primer binding sequences 1, 2 and 3 should be at least four or more bases to bind with template DNA. Since the primer was well combined with template DNA in the size of 8-30 bases, the number of bases preferably ranges from 8 to 30. The 'front sequence from mutation' is a sequence toward 5' of the mutation to be examined. The 'mutation sequence' is a sequence corresponding to a mutation to be examined. Substitution, insertion and deletion of bases may occur, wherein the number of bases is generally 1 and may be two

or more. The 'sequence behind mutation' is a sequence toward 3' of the mutation sequence.

Preferably, the total number of bases of the front sequence from mutation and behind mutation is one or more. The fragments resulting from restriction enzyme cleavage should include mutation sequences, and the size of the fragment should range from 2 to 32 bases. Preferably, the size is 12 bases. The reason the size of cleaved fragments is limited is that there is a good result in case of the favorable size of fragments in MALDI-TOF analysis. The above number of bases in fragments is preferable because the fragment having a size out of the range is too large to examine mutations by measuring the molecular weight using the MALDI-TOF analysis. Since the two restriction enzymes recognize the same sites, it is preferable that one restriction enzyme is not activated while the other restriction enzyme reacts with the amplified product. When the amplified fragments are cleaved with restriction enzymes, reaction may be performed consecutively at different temperatures in consideration of the optimum temperatures of two restriction enzymes.

Example 1. Mutation of the 2741$^{st}$ base of the 4$^{th}$ intron of human maspin gene

Mutation of the 2741$^{st}$ base (rs1509477; the 61001755th base of chromosome No. 18) of the 4$^{th}$ intron of human maspin (serpinb5) gene that is known as cancer metastasis inhibition gene is examined.

**1. PCR amplification and restriction enzyme cleavage**

The sequence (5' → 3') of template DNA is as follows.

GTTTCACTTGATAAAGCAATAAAATGCTATTCAcAGCTGCATGAGGCT

ACACCCTTCTTTTGAATGCAG (SEQ ID NO: 1)

The underlined sequences are sites where the following primers 1 and 2 are hybridized. The bases represented by small letters are 'mutation sequence'.

Primer 1. 5' – TCACTTGATAAAGCAATAAAAggatgGCTATTCA - 3' (34mer) (SEQ ID NO: 2)

Primer 2. 5'- CATTCAAAAGAAGGGTGTAGCCTCATGC – 3' (28mer) (SEQ ID NO: 3)

The sequences represented by small letters are recognition sequences of Fok1 and BstF5I.

PCR buffer (1x), 2mM of $MgSO_4$, 200mM of dNTP, Platinum Taq Polymerase (Invitrogen, 10966-026) 0.315U, 0.5 μM of primer 1 and 0.5 μM of primer 2, and 36ng of genomic DNA were added to be 18μl of the total reaction volume. Then, the PCR reaction was performed under the following condition.

94°C, 2min.

94°C, 15sec. 55°C, 15sec. 72°C, 30sec. (10 cycles),

94°C, 15sec. 60°C, 15sec. 72°C, 30sec. (35 cycles)

The genomic DNA was isolated from blood and purified. For example, 'SDS/protease K' method (Maniatis, Molecular Cloning, A laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) or QIAamp DNA Mini Kit 250 (Qiagen 51106) could be used in isolation of DNA from blood. When the concentration of DNA is low, the DNA can be concentrated by the following method. First 1/10 volume of 3 M Sodium acetate (pH 5.3) and 2.5 volume of ethanol were

added to DNA solution and gently mixed. The resulting solution was left at -20°C for more than 1 hour, and then centrifuged at 4°C, 13000rpm for 15 minutes. After the supernatant was removed, 70% ethanol was added and the resulting solution was centrifuged at 4°C, 13000rpm for 10 minutes. Then, ethanol was dried, and desired volume of distilled water was added to the resulting solution.

The sequence of fragments obtained from the PCR is as follows (5'→ 3').

TCACTTGATAAAGCAATAAAAggatgGCTATTCA[C/T]AGCTGCATGAG GCTACACCCTTCTTTTGAATG (SEQ ID NO: 4)

AGTGAACTATTTCGTTATTTTcctacCGATAAGT[G/A]TCGACGTACTCC GATGTGGGAAGAAAACTTAC (SEQ ID NO: 5)

The sites represented by small letters are sequences recognized by Fok1 and BstF5I, the underlined sites are sequences of fragments generated by restriction enzyme cleavage, and the bases represented by brackets are 'mutation sequences'. To the reactant were added FokI (NEB R109L) 1U, BstF5I (NEB, V0031L) 1U, 50mM of potassium acetate, 20mM of Tris-acetate, 10mM of magnesium acetate, 1mM of DTT (pH 7.9 @ 25°C). The resulting solution was reacted at 25°C for 2 hours, and consecutively at 45°C for 2 hours.

For optimization of enzyme reaction, the amplified products were reacted with FokI and BstF5I at 25°C, 37°C, 45°C, 55°C and 65°C. As a result, 70% of enzyme reaction proceeded at 25°C, and more than 90% enzyme reaction proceeded at 37°C in case of FokI. In case of BstF5I, the enzyme reaction didn't proceeded at 25°C. Accordingly, the products were preferably reacted first at 25°C where only FokI could

react, and then at over 37°C where BstF5I could react.

### 2. Purification and Desalination

Preferably, DNA fragments were purely isolated from the above solution treated with restriction enzymes, and then the molecular weights of the fragments were measured. For example, Nucleave Genotyping Kit (Variagenics, USA) might be used. 70μl of a conditioning reagent (1M TEAA) was added to the restriction enzyme reaction solution, and left for 1 minute. 70μl of the conditioning reagent and 90μl of the above mixed solution were added to a Sample Preparation Plate, and then 85μl of a wash reagent (0.1M TEAA) was five times passed through the Sample Preparation Plate. The Sample Preparation Plate was centrifuged at 1000rpm for 5 minutes. Thereafter, the Sample Preparation Plate was placed on a Collection Plate, and then 60μl of an elution reagent (60% isopropanol) was added thereto and passed. When the effluent solution was collected in the Collection plate, the Collection Plate was dried at 115°C for 75 minutes.

### 3. MALDI-TOF Mass Spectrometry

6μl of MALDI matrix (22.8mg ammonium citrate, 148.5mg hydroxypicolinic acid, 1.12ml acetonitrile, 7.8ml H20) was added to the Collection Plate, and then 4μl of mixture of the MALDI matrix and effluent solution was placed on an Anchor chip plate of MALDI-TOF (Biflex IV, Bruker). It was dried at 37°C for 30 minutes, left at room temperature to be cooled for a while, and then subjected to MALDI-TOF analysis. The analysis method follows the MALDI-TOF manual.

When the 2741st base of the 4th intron is normal (C/C), the molecular weight of fragments obtained after enzyme cleavage is 2135.4 D (7mer) and 4078.6 (13mer) D (see Figs. 1 and 2). When the 2741st base of the 4th intron is hetero (C/T), the molecular weight of fragments is 2135.4 D, 2150.4 D (7mer) and 4078.6 D and 4062.6 D (13mer) (see Figs. 3 and 4). When the 2741st base of the 4th intron is all changed into T (T/T), the molecular weight of fragments is 2150.4 D (7mer) and 4062.6 D (13mer) (see Figs. 5 and 6).

Example 2. Mutation of the 3597th base (rs1396782; 61002611th base of chromosome No. 18) of the 4th intron of human maspin (serpinb5) gene known as human cancer metastasis inhibition gene

The sequence of template DNA is as follows.

CTGGAGTATTATCCTTGCAGGCTTGATATGAAGcTTGAAATTTCTCCC CAAAGAGATTTAGTTAACAGGCAAA (SEQ ID NO: 6)

The underlined sequences are sites where the following primers 3 and 4 hybridize. The mutation represented by a small letter is a 'mutation sequence'.

Primer 3. 5' GAGTATTATCCTTGCAGGCTTggatgATATGAAG 3' (34mer) (SEQ ID NO: 7)

Primer 4. 5' – GCCTGTTAACTAAATCTCTTTGGGGAGAA 3' (29mer) (SEQ ID NO: 8)

The sites represented by small letters in the above primers are sequences that do not exist in template DNA, but Fok1 and BstF5I recognize them. The experimental method including the PCR reaction is the same as that of Example 1.

The sequences of fragments obtained through the PCR are as follows (5'→3').

GAGTATTATCCTTGCAGGCTTggatgATATGAAG[C/T]TTGAAATTTCTC CCCAAAGAGATTTAGTTAACAGGC (SEQ ID NO: 9)

CTCATAATAGGAACGTCCGAAcctacTATACTTC[G/A]AACTTTAAAGAG GGGTTTCTCTAAATCAATTGTCCG (SEQ ID NO: 10)

The sites represented by small letters in the above sequences are restriction enzyme recognition sequences, the underlined sites are sequences of fragments obtained from restriction enzyme cleavage, and the bases represented by brackets ([ ]) are 'mutation sequences'. To the reactant were added FokI (NEB R109L) 1U, BstF5I (NEB, V0031L) 1U, 50mM of potassium acetate, 20mM of Tris-acetate, 10mM of magnesium acetate, 1mM of DTT (pH 7.9 @ 25°C). The resulting solution was reacted at 25°C for 2 hours, and consecutively at 45°C for 2 hours.

When the 3597$^{th}$ base of the 4$^{th}$ intron is normal (C/C), the molecular weight of fragments obtained from enzyme cleavage is 2209.4 D (7mer) and 3988.6 D (13mer) (see Fig. 7). When the 3597$^{th}$ base of the 4$^{th}$ intron is hetero (C/T), the molecular weight of fragments is 2209.4 D, 2224.4 D (7mer), and 3988.6 D and 3972. 6 D (13mer) (see Fig. 8). When the 3597$^{th}$ base of the 4$^{th}$ intron is all changed into T (T/T), the molecular weight of fragments is 2224.4 D (7mer) and 3972. 6 D (13mer) (see Fig. 9).

[EFFECTS OF THE INVENTION]

In an embodiment of the present invention, the analysis misled by errors in the conventional method for detecting mutations may be identified, and inability of the tests

to detect mutations resulting from deletion or insertion can be solved.

[CLAIMS]

[Claim 1]

A method for detecting a mutation, comprising:

a) amplifying a target polynucleotide using a forward primer and a reverse primer;

b) cleaving the amplified target polynucleotide with restriction enzymes having different optimum temperatures; and

c) measuring the molecular weight of the cleaved fragments.

[Claim 2]

The method according to claim 1, wherein the forward primer comprises a primer binding sequence 1, a restriction enzyme recognition sequence and a primer binding sequence 2.

[Claim 3]

The method according to claim 1 or 2, wherein the forward primer is a primer set forth in SEQ ID NO: 2 or 7.

[Claim 4]

The method according to claim 1, wherein the reverse primer is a primer set forth in SEQ ID NO: 3 or 8.

[Claim 5]

The method according to claim 1, wherein restriction enzyme treatments step is performed under a condition that one enzyme reaction is not activated while the other enzyme reaction is performed.

[Claim 6]

The method according to claim 1 or 5, wherein the restriction enzymes are a restriction enzyme having a low optimum temperature selected from the group consisting of Fok1, Bbv I, Bsg I, Bcg I, Bpm I, BseR I and Bae I, and a restriction enzyme having a high optimum temperature selected from the group consisting of BstF5 I, Taq I, BsaB I, Btr I, BstAP I, Fau I, Bcl I, Pci I and Apo I.

[Claim 7]

The method according to claim 1, wherein the fragments cleaved by the restriction enzymes comprises a mutation sequence

[Claim 8]

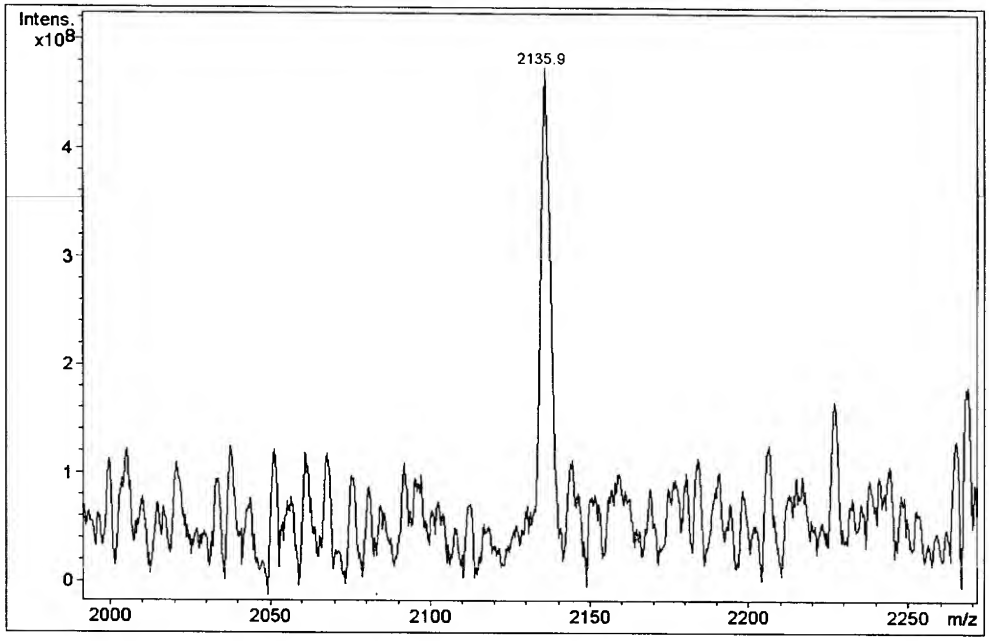
The method according to claim 1 or 7, wherein the fragment cleaved by the restriction enzymes has the size of bases ranging from 2 to 32

[Claim 9]

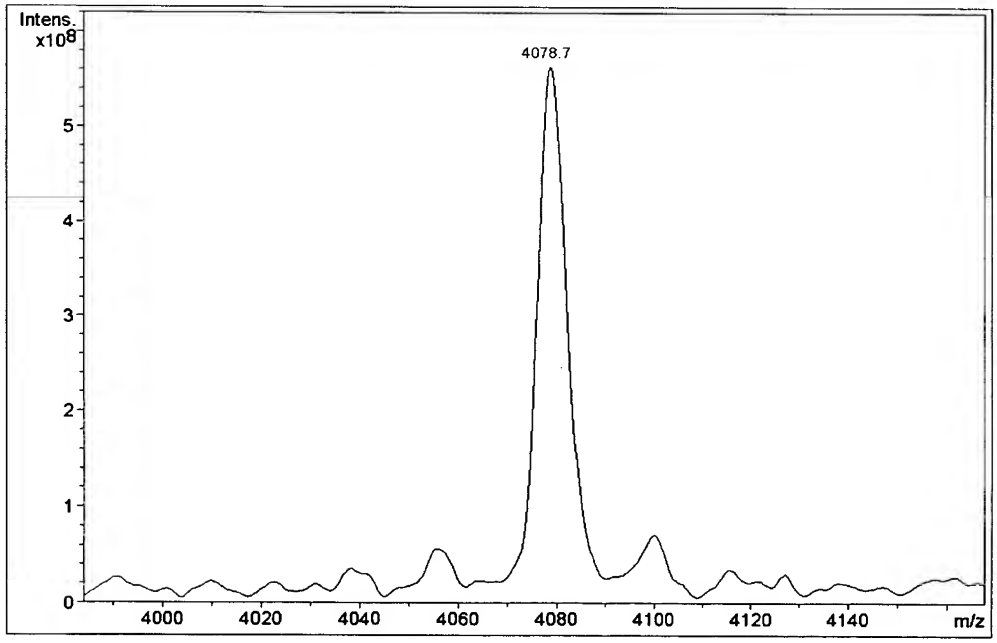
The method according to claim 8, wherein the fragment has the base size of 12.

[DRAWINGS]

[FIG. 1]




[FIG. 2]



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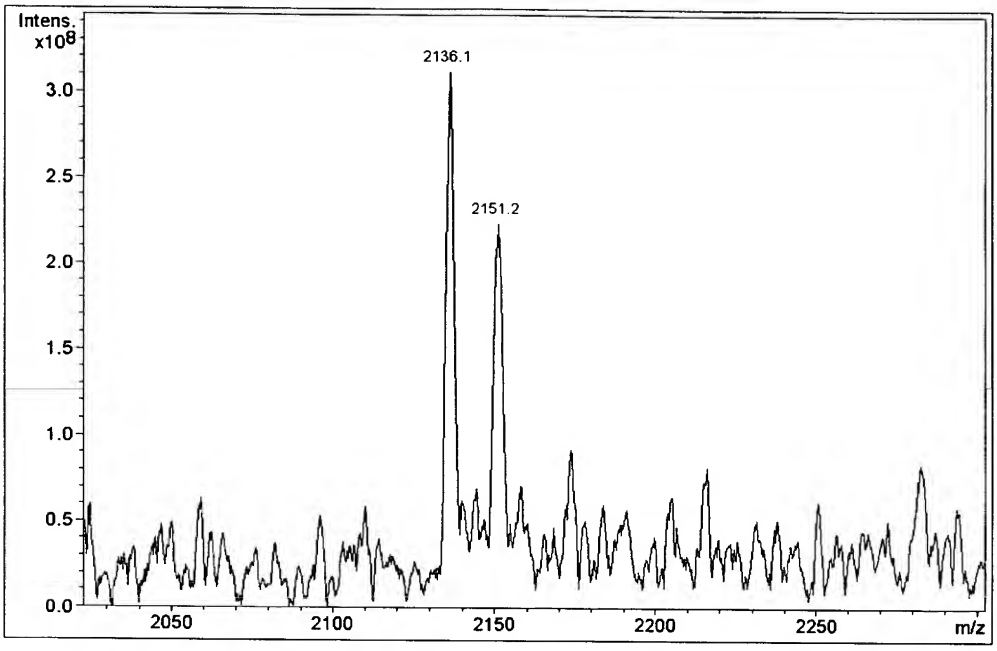
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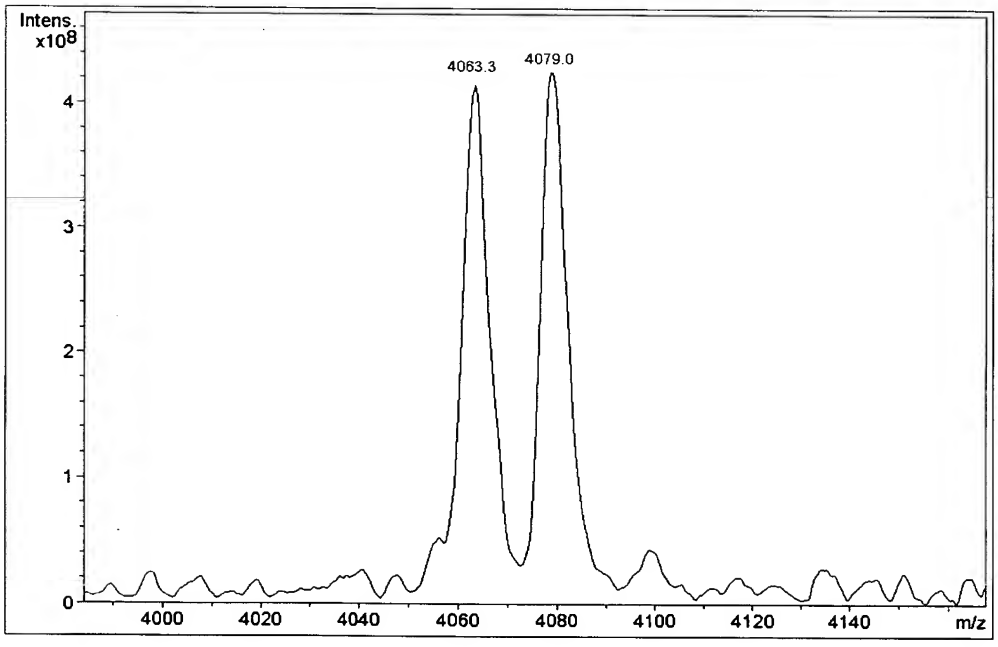
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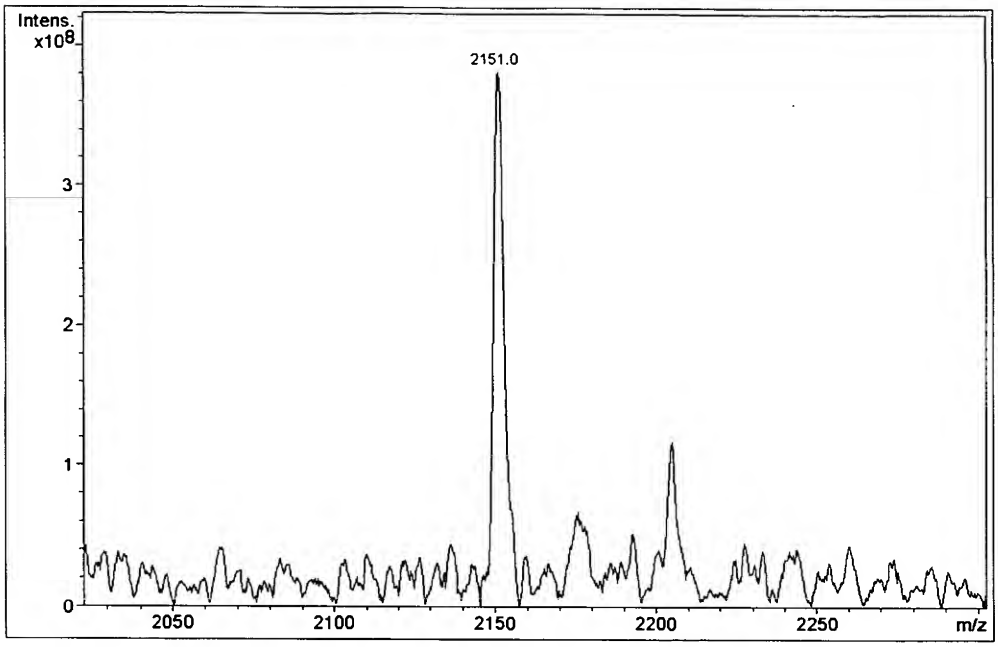
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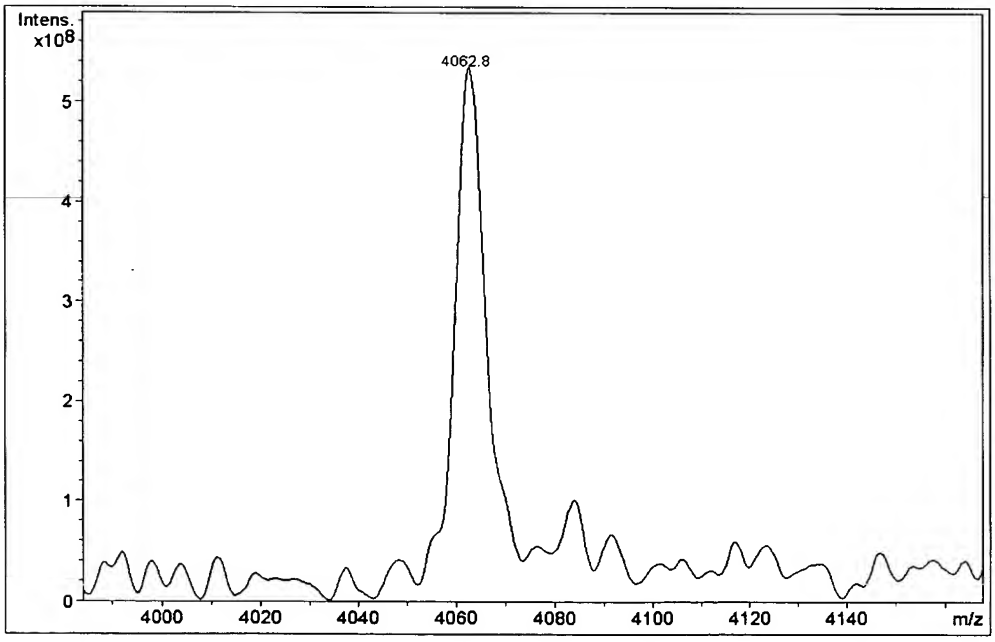
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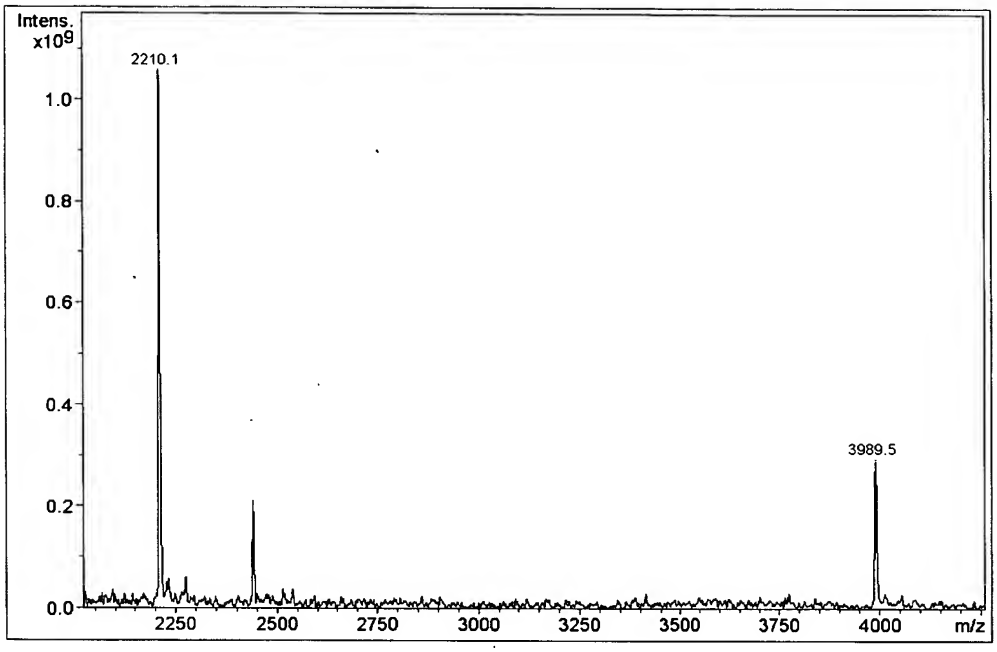
[FIG. 7]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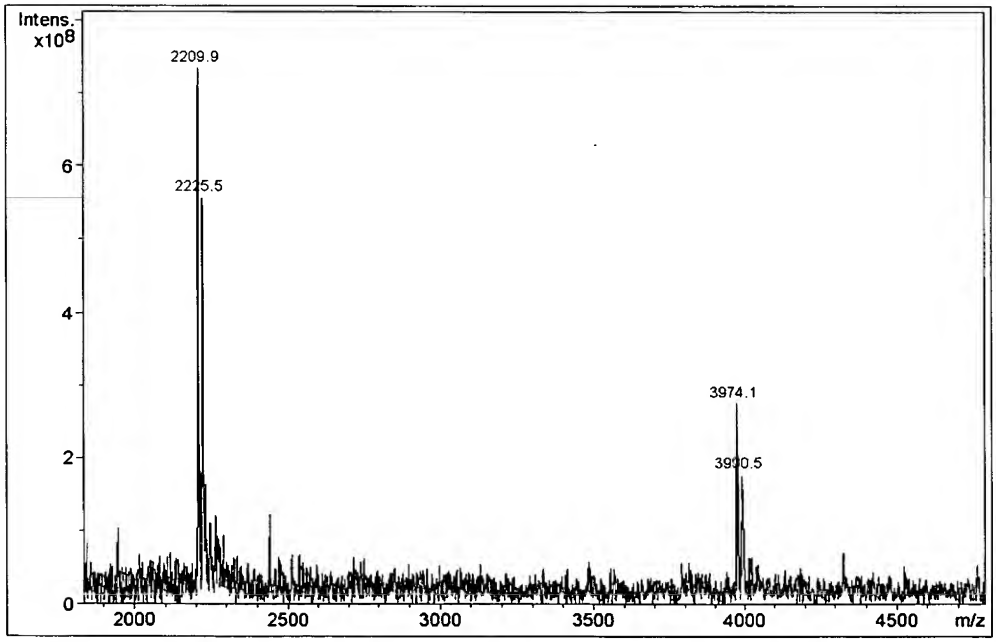
[FIG. 8]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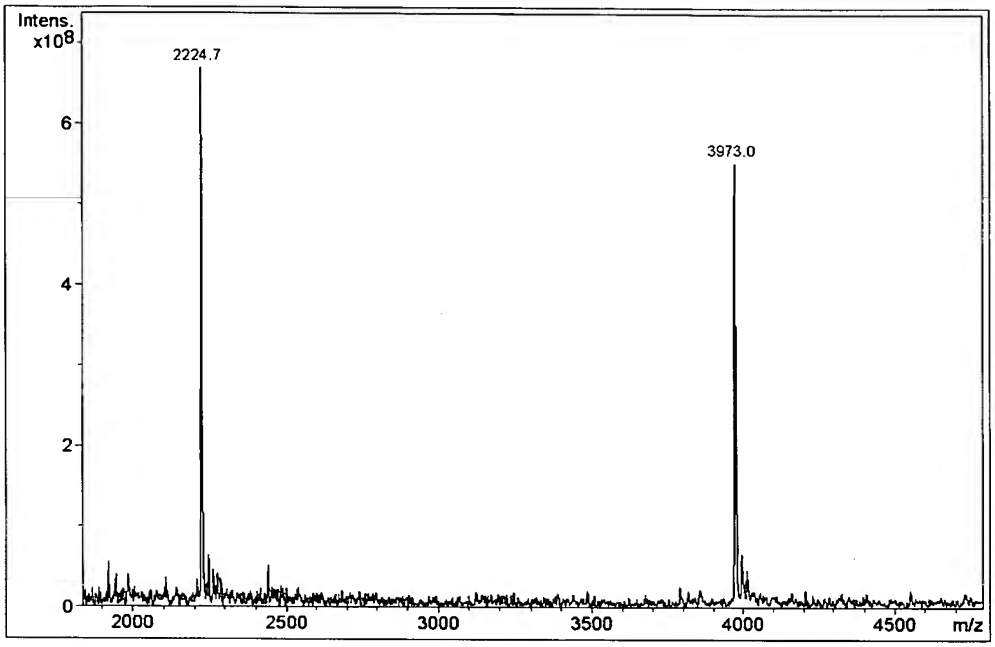
[FIG. 9]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<211> 72
<212> DNA
<213> Artificial Sequence
<220>
<223> Resulting PCR Fragment
<400> 10
ctcataatag gaacgtccga acctactata cttcgaaact ttaaagaggg gtttctctaa 60
atcaattgtc cg 72